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Potentiation of $GABA_A$ Receptor in Cultured Mouse Hippocampal Cells by Brain-Derived Peptide Mixture Cerebrolysin

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Summary

Application of Cerebrolysin (0.1 μg per 1 ml) by a fast microperfusion system induced an inward current of 0.2 to 1 nA in all neurones from newborn mouse hippocampi held at −30 mV membrane potential. Cerebrolysin-induced currents were reduced by the $GABA_A$ antagonist bicuculline (2 μM) by 65 %, by the NMDA antagonist aminophosphovaleric acid (APV, 10 μM) by 27 %, and by the non-NMDA antagonist cyanonitroquinoxalinedione (CNQX, 10 μM) by 20 %. Cerebrolysin dialyzed through a 3.6 kD gut did not induce any transmembrane current but potentiated the response induced by GABA (10 μM) to 135 %. We conclude that, in addition to amino acids which activate $GABA_A$, NMDA and non-NMDA receptors, Cerebrolysin also contains a peptide which potentiates the $GABA_A$ receptor response.

Key words

Mouse hippocampal neurones – Cerebrolysin – $GABA_A$ receptor – NMDA receptor – Non-NMDA receptor – Patch clamp

Introduction

Biologically active peptides in the central nervous system, that are released from peptidergic neurones or as cotransmitters with classical neurotransmitters, have the capability to activate a new class of receptors or to modulate synaptic transmission (Hökfelt 1991). Fragments of synaptic vesicle membrane proteins have also been identified to exert a modulatory role (Chartel *et al.* 1994). Cerebrolysin is a brain-derived nootropic peptide mixture that is produced by standardized enzymatic hydrolysis of lipid-free pig brain proteins. It is clinically used in disorders where injured neurones should be protected from further damage, for example in the therapy of neurodegenerative diseases such as senile demention of the Alzheimer type (Ruther *et al.* 1994). In animal experiments, Cerebrolysin was found to facilitate learning and the memory processes in rats (Paier *et al.* 1992). The molecular mechanism of the complex beneficial action of Cerebrolysin is not yet known. The aim of the present study was to determine what is the direct effect of Cerebrolysin application to neurones in cell culture and whether it can modulate the function of some neurotransmitter receptors.

Material and Methods

Experiments were performed on cultured hippocampal nerve cells prepared from 16 to 18-day-old mouse BALB/c embryos (Mayer and Vyklický 1989). Trypsinated hippocampi were dissociated and plated onto a confluent glial feeder layer prepared in advance from newborn mice. The growth medium contained Eagle's minimal essential medium (MEM), 5 % horse serum and a nutrient supplement composed of transferrin, insulin, selenium, corticosterone, triiodothyronine and progesterone (Guthrie *et al.* 1987). Cell division was suppressed using the metabolic inhibitor 5-fluoro-2'-deoxyuridine. Nerve cells were used for electrophysiological experiments after 5–14 days in culture. Newborn mice and donor mother mice were killed by cervical dislocation.

During the experiments, the dishes with cell cultures were continuously superfused with an extracellular solution of the following composition (in mM): NaCl 160, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 1, glucose 10, HEPES 10, pH adjusted to 7.3 with 1 M NaOH. Tetrodotoxin (TTX) 0.5 μM was routinely added to the

superfusing solution to block voltage-dependent sodium channels and synaptic potentials.

Membrane currents were recorded with a List EPC-7 amplifier in the whole-cell configuration (Hamill *et al.* 1981). Patch electrodes used for whole-cell recording were filled with an intracellular solution containing (in mM): CsCl 140, EGTA 5, $CaCl_2$ 0.5, $MgCl_2$ 1, HEPES 10, pH adjusted with CsOH to 7.2. Electrodes were pulled from glass tubes with a 1.65 mm outer diameter. The tip of the pipette had an outer diameter of about 3 μm and the pipette resistance was 3–10 MΩ. Tight-seal whole-cell recordings were always performed using 50–60 % series resistance compensation. Data were stored in digital form using a modified digital-audio processor (Sony PCM-501ES, frequency 20 kHz) and a video tape recorder.

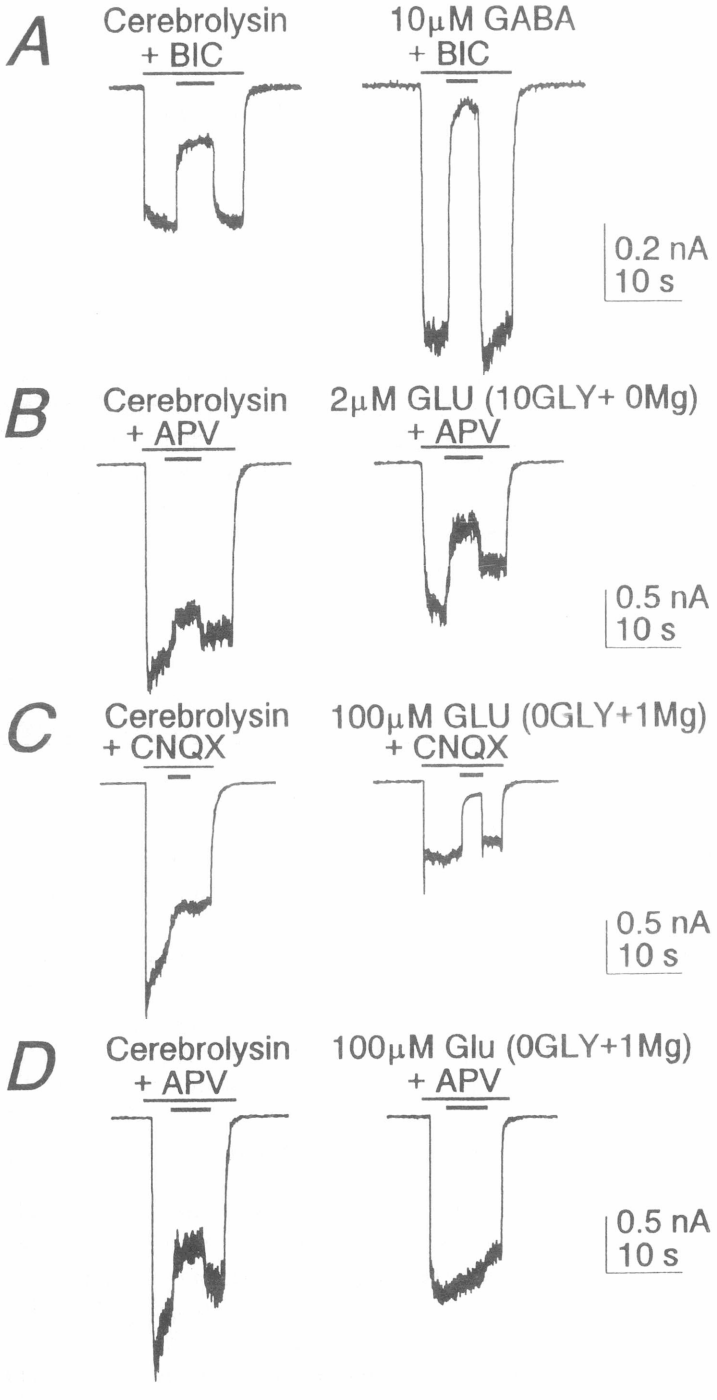

Fig. 1
Inhibition of current induced by Cerebrolysin (0.1 μg/ml) by 2 μM bicuculline (BIC) (A), and of currents induced by Cerebrolysin (0.2 μg/ml) by 10 μM aminophosphovalerate (APV) in the presence of 10 μM glycine (GLY) and absence of Mg^{2+} (B), by 10 μM of cyanonitroquinoxalinedione (CNQX) (C), and by 10 μM APV in the absence of glycine and presence of Mg^{2+} (D). For comparison, the inhibition of currents induced by specific agonists, glutamate (GLU) and γ-aminobutyric acid (GABA) is also shown as the second record from the same cell in A, B, C and D. Temperature 22 °C.

Cerebrolysin and the drugs tested were applied using a fast perfusion system (Mayer and Vyklický 1989) consisting of a peristaltic pump and an array of ten glass tubes, each approximately 400 μm in diameter. Movement of the glass tube array and solution application were achieved by a microcomputer-controlled system (for details see Vyklický *et al.* 1990). A complete change of the solution

around the neurone varied between 20–60 ms depending on the speed of the solution expelled and on the arborization of the neurone under study.

Amino acid-free Cerebrolysin was prepared by extensive dialysis of crude Cerebrolysin overnight through a 3 600 D gut against a buffer (10 mM HEPES, pH 7.3) at 4 °C.

Cerebrolysin (EBEWE Austria), glutamate, γ-aminobutyric acid, glycine, bicuculline, aminophosphovaleric acid (Sigma), cyanonitroquinoxalinedione (Tocris Cookson) and TTX (Calbiochem) were used. Experiments were performed at 22–25 °C. Data are expressed as the mean ± S.D.

Results

All neurones (n=32) responded to 3 s application of Cerebrolysin (0.1 μg per 1 ml medium) by 0.2–1 nA inward current. The amplitude of this current was equal to 40.2±9.8 % of the control current response induced by 10 μM γ-aminobutyric acid (GABA). Using specific inhibitors for three neurotransmitter receptors, the currents induced by Cerebrolysin (0.2 μg/ml) were further studied (Fig. 1). When the GABA receptor type A ($GABA_A$) antagonist bicuculline (2 μM; Akaike *et al.* 1985) was tested, the Cerebrolysin-induced currents decreased by 65 % whereas the control currents evoked by 10 μM GABA were reduced by 94 % (Fig. 1A, 4 neurones). In the presence of 10 μM glycine and in the absence of Mg^{2+}, the specific NMDA antagonist aminophosphovaleric acid (APV, 10 μM; Honoré 1989) that inhibited control currents induced by 2 μM glutamate by 48 %, also decreased the Cerebrolysin-induced current, but only by 27 % (Fig. 1B, 6 neurones). Without glycine and with 1 mM Mg^{2+} in the bath, high glutamate (100 μM) induced APV-insensitive currents which were inhibited by the specific non-NMDA antagonist cyano-nitroquinoxalinedione (CNQX, 10 μM) by 95 %. Under the same conditions 10 μM CNQX inhibited Cerebrolysin-induced currents by 20 % but these currents were also sensitive to the NMDA antagonist APV (10 μM) which decreased them by 25 % (Fig. 1C,D). This observation might be explained by the presence of glycine in Cerebrolysin, which activates the modulatory glycine-binding site of the NMDA receptor.

Cerebrolysin is a brain-derived peptide preparation which contains peptides of molecular weight below 10 000 D and free amino acids. All the above mentioned effects of Cerebrolysin could be ascribed to amino acids present in Cerebrolysin. It was of interest to know what is the effect of the peptide component of Cerebrolysin. Cerebrolysin which was dialyzed overnight through a 3 600 D gut did not induce any transmembrane current when used in the concentration of 0.4 μg/ml. Dialyzed Cerebrolysin, applied prior to GABA application or simultaneously with GABA, increased the amplitude of the current induced by 10 μM GABA to 134.8±13.2 % (Fig. 2, 8 neurones). The effect of dialyzed Cerebrolysin was reversible and could be removed after 1 min of intensive washing. No effect of dialyzed Cerebrolysin on the glutamate responses was observed. These data strongly suggest that, besides amino acids activating $GABA_A$, NMDA and non-NMDA receptors, Cerebrolysin contains some endogenous factor(s) modulating the $GABA_A$ neuronal receptor.

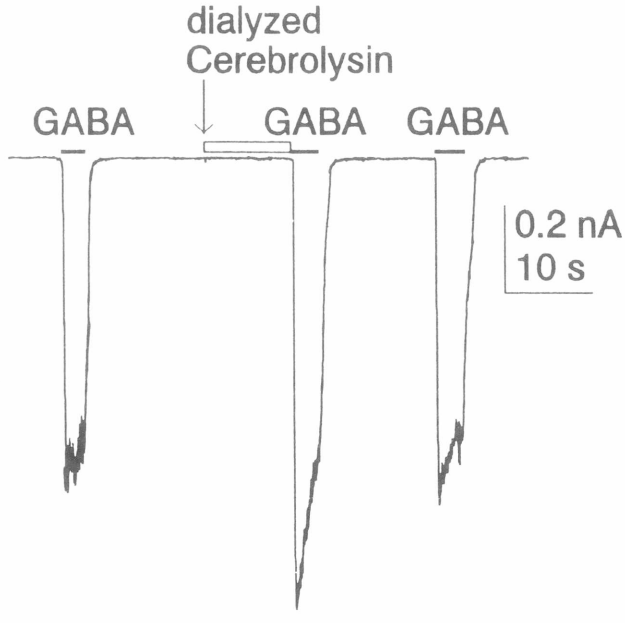

Fig. 2
The potentiating effect of dialyzed Cerebrolysin (0.4 μg/ml) on currents induced by 10 μM GABA. Dialyzed Cerebrolysin was applied for 20 s prior to GABA application. Note recovery of the GABA response after 30 s of washing.

Discussion

Cerebrolysin applied directly to the neurones activated receptors for amino acids ($GABA_A$, NMDA and non-NMDA receptor). This was not surprising, because Cerebrolysin contains 85 % of free amino acids and 15 % of low molecular-weight peptides (below 10 000 D) and its effect was removed by dialysis. Various neuropeptides, but not amino acids, when peripherally administrated, are able to penetrate through the blood-brain barrier (Banks and Kastin 1985) and to modulate central nervous functions (De Wied and van Ree 1971). The main active substance of Cerebrolysin is thus apparently its peptide component.

Dialyzed Cerebrolysin, which was devoid of amino acids and contained peptides with molecular weights above 3 000 D, did not induce any

transmembrane currents but potentiated responses mediated by the $GABA_A$ receptor. The $GABA_A$ receptor, which forms a complex with the chloride ionic channel, contains a number of modulatory sites capable of binding exogenous facilitatory agents such as benzodiazepines (Squires and Braestrup 1977, Möhler and Okada 1977), pentobarbital (Akaike *et al.* 1990) and avermectins (Sigel and Baur 1987, Krůšek and Zemková 1994). The specific benzodiazepine (BZD) receptor is an integral part of the $GABA_A$ receptor (Squires and Braestrup 1977, Möhler and Okada 1977), and drugs acting on this receptor (for example diazepam) exhibit anxiolytic, anticonvulsant and antidepressant effects (Martin 1987). The potentiating effect of dialyzed Cerebrolysin is of interest from the viewpoint of the ongoing discussion whether endogenous ligands exist which could naturally react with the BZD receptor (for review see De Robertis *et al.* 1988, Klotz 1991). Various putative candidates have been hypothesized since the discovery of BZD receptors in 1977. Costa *et al.* (1983) isolated a polypeptide named diazepam-binding inhibitor from the rat brain that competitively inhibited ^{3}H-diazepam binding in micromolar concentrations. This protein has a M_r of 11 000 D, contains 105 amino acids and was found in 10–25 μM concentration in the brain. Other substances which have been isolated so far are BZD-like molecules, often benzodiazepine metabolites (Guentert 1984), which are thermostable and resistant to proteases, and probably of plant or bacterial origin (Costa *et al.* 1983, Sangameswaran and De Blas 1985). It would therefore be of great importance to determine whether some peptide component of Cerebrolysin contains the BZD-like molecule responsible for $GABA_A$ receptor potentiation. Alternatively, it is not the BZD-like molecule but the endogenous peptide itself which is responsible for this effect of Cerebrolysin. Whether stimulation of $GABA_A$ receptor may have relevance to the therapeutic effect of Cerebrolysin remains to be clarified.

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